ACCURATE *IN VITRO* METHODS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING OF BACTERIAL PATHOGENS

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ABSTRACT

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Title: Accurate methods for *in vitro* antimicrobial susceptibility testing of bacterial pathogens

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Respiratory infections caused by the opportunistic bacterial pathogen *Pseudomonas aeruginosa* are a common cause of mortality in patients with cystic fibrosis (CF). These infections are difficult to eradicate with conventional antibiotic treatment as *P. aeruginosa* is often innately resistant to many antibiotics. In clinical practice, diagnostic labs test *P. aeruginosa* strains *in vitro* (outside of a living organism) in order to determine an appropriate antibiotic to prescribe to each patient. However, these test results can be misleading because antibiotic resistance is highly dependent on the chemical environment in which the bacteria grow. Therefore, it is essential that *in vitro* models used to assess antibiotic resistance resemble *in vivo* growth conditions as closely as possible. In this thesis, I compared the antibiotic resistance of *P. aeruginosa* in two growth conditions: within a complex media commonly used in diagnostic labs, and a chemically defined synthetic sputum media (SCFM2) constructed to closely mimic the lung fluid of cystic fibrosis patients. I tested *P. aeruginosa* in both media, using three clinically relevant antibiotics (tobramycin, gentamicin, and polymyxin B), and found that the bacteria produce significantly different antimicrobial susceptibility profiles depending on the media in which they are cultured. Ultimately, the data presented here have the potential to help guide diagnostic labs to create more accurate *in vitro* models to predict more effective treatments for individual patients.

Section One: General Information

Background: Cystic Fibrosis

What is Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder that affects many parts of the body. It is the most prevalent fatal genetic disorder in the United States (O'Sullivan et al., 2009), and it is thought to have arisen in Europe roughly 5000 years ago (Busch et al., 1990). The disease can vary greatly in severity, though nearly all patients suffer from respiratory problems. These respiratory symptoms almost always result in bacterial infections of the lungs and airways (Govan et al., 2007), which can be extremely difficult to effectively treat through conventional antibiotic therapy. Because of this, the most common cause of mortality of CF patients is complications resulting from chronic bacterial lung infections. Therefore, advancements in CF research are often focused on understanding more about and improving treatments of these chronic infections.

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder caused by mutation of the cystic fibrosis transmembrane regulator (CFTR) gene (Sosnay et al., 2016). "Autosomal" refers to the fact that the gene is on a non-sex chromosome, meaning that it is not carried on female X chromosomes or male Y chromosomes. "Recessive" means that a patient must have two mutant copies of the gene in order to have the disease. Generally speaking, sexual organisms have two alleles of every gene, one transmitted from the mother and one from the father. This means that CF patients must receive a mutation from both of their parents. However, since it is a recessive disorder, it is not uncommon for patients to have very few effected family members.

The mutation responsible for CF is thought to have originated in Europe, which is supported by both historical and scientific accounts . CF is most prevalent in people of

Northern European decent, and is the most common fatal genetic disorder in the United States, occurring once per every 3000 live births (O'Sullivan et al., 2009). However, the occurrence of CF in specific countries can vary. In Romania, for instance, 1 in every 2000 babies is born with CF, and in Canada, 1 in every 2500 (Farrell et al., 2008). Other ethnic groups are also affected by CF, but to a lesser degree. It's estimated that 1 in every 4000-10,000 Latin American children, and 1 in every 15,000-20,000 African American children are born with CF (Macri et al., 1991; Phillips et al., 1995). The disorder is extremely rare in Africa and Asia, by contrast (Padoa et al., 1998; Singh et al., 2015). In Japan, for example, the reported rate of incidence is 1 in every 350,000 births (Yamashiro et al., 1997). In the United States, 1 in every 3000 babies are born with CF, and it is estimated that 1 in 25 Americans are carriers of the genetic mutation (O'Sullivan et al., 2009). Men and women are affected by CF equally, though symptoms of reproductive organs are different between the sexes.

The name of the gene, CFTR, stands for "Cystic Fibrosis Transmembrane Conductance Regulator." Like many genes, the name is an indicator of the gene's function. The transmembrane conductance regulator is a protein found on cell membranes that functions as a chloride channel (Sosnay et al., 2016). It's function is regulating the transport of chloride ions in and out of epithelial cells. A secondary function of this protein is inhibiting sodium transport from sodium channels. Therefore, mutations in this gene cause dysfunction in the transport of salt and fluids across cell membranes. One consequence of this is the presence of abnormally high levels of salt on the patient's skin, which is one of the only symptoms of CF that can be detected without specialized testing (Gibson et al., 1959). Mutations in the CFTR gene cause a variety of symptoms, and a

wide array of organs can be affected (di Sant'Agnese et al., 1956; Kaplan et al., 1968). One of the milder symptoms of the disease is unusually high levels of salt in the patient's sweat. Due to the harmlessness of the symptom and its relative ease of detection, salt levels in the sweat is now the main method that doctors use to diagnose CF.

However, not all symptoms of the disease are benign. Many vital organs within the body—such as the lungs, pancreas, and the reproductive organs in males—are greatly impacted by the disorder (di Sant'Agnese et al., 1956; Kaplan et al., 1968). In males, abnormal secretions and fluid transport can cause complications in the development of the vas deferens, which is responsible for transporting sperm to ejaculatory ducts prior to ejaculation. This obstruction often leads to the absence of the vas deferens in male CF patients, leading to infertility in 97% of males (Chotirmall et al., 2009). This does not always leave male CF patients sterile, however, because sperm production is not always affected. As a result, some men with CF can have children through medical assistance. In other cases however, some men with CF do have a lack of sperm production or have abnormal sperm and are unable to have children. Infertility only effects 20% of women with CF. This infertility is often caused by thickened cervical mucus, or in rarer cases, malnutrition as a result of the disease ("Cystic Fibrosis Foundation: Reproductive Health and Fertility," 2016).

Thickened mucus is a key symptom of CF. The thickened mucus is a direct result of fluid transport problems resulting from the aforementioned mutations in the CFTR gene. This mucus has a profound effect on pancreatic function, causing a variety of complications (di Sant'Agnese et al., 1956). The pancreas' main function is producing and releasing digestive enzymes into the intestine to break down food after it has left the

stomach. Thickened secretions in and around the pancreas can obstruct pancreatic ducts, which is where these enzymes are released from the pancreas. In severe cases, these ducts can be completely clogged (di Sant'Agnese et al., 1956). In addition to damage to the pancreas, this also prevents food from being broken down after passing through the stomach. Since the enzymes are either absent or insufficient in number, the nutrients from food are not able to be extracted and absorbed through the intestines. This symptom, a condition known as meconium ileus, is one of the earliest signs of CF, occurring in 5-10% of affected newborns (Mitchell et al., 2007). While this obstruction can be relieved using a variety of procedures, if untreated it can result in severe malnutrition, inability to pass feces, abdominal pain, and, eventually, death.

In modern cases of CF, however, complications of the lung are by far the most pressing concern. The thick secretions resulting from abnormal functioning of transmembrane conductance regulators causes a dramatic thickening of the mucus lining of the lungs (Lieberman et al., 1960). In healthy people, this mucus is thin and fluid. Its function is to trap bacteria and other potentially harmful particles in order to prevent lung damage and infection (Lillehoj et al., 2002). This mucus works in conjunction with cilia, tiny hair-like structures that sweep away particles trapped in the mucus, keeping the lungs and airways clear. In patients with CF, respiratory mucus, like other bodily secretions, is abnormally thick. While the mucus is still able to trap particles that enter the lung, the extreme thickness and viscosity makes it impossible for cilia to sweep the mucus—and the particles trapped within it—out of the lungs (Lieberman et al., 1960). As a result, the lungs of CF patients become increasingly filled with mucus, obstructing breathing and leading to violent fits of coughing. Other airways, such as the sinuses and trachea also

become clogged with respiratory mucus, with hinders breathing even further. Patients with CF must undergo regular therapies—often multiple times a day—to clear airways in order to retain breathing function (Bradley et al., 2005; Flume et al., 2009).

However, obstructing the patient's ability to breathe is not the only complication that occurs inside the lungs of patients with CF. Since the lung cilia are unable to naturally clear the patient's lungs, trapped bacteria begin to multiply very rapidly inside the lungs. Respiratory mucus, incidentally, provides a rich, nutritious medium for many bacteria to thrive. While physical airway clearance techniques help CF patients to expel lung mucus to a certain degree, clearing bacteria is a much more difficult task. As a result, nearly all patients with CF suffer from chronic lung infections. These infections are responsible for 80% of CF patient deaths, making chronic lung infections the leading cause of mortality (O'Sullivan et al., 2009). Because of this, modern CF research is centered around the prevention and treatment of chronic bacterial lung infections.

The History of Cystic Fibrosis Research

Although the disorder likely emerged as early as 3000 BCE (Busch, 1990), CF was not recognized by modern medicine until the mid-twentieth century (Andersen, 1938). However, recorded knowledge of the disorder can be found in various texts throughout human history, indicating that our ancestors recognized the symptoms of CF long before scientific evidence was able to corroborate.

Prior to medical research on the disorder, children and infants with CF typically died of complications related to malfunctions of the pancreas, which is responsible for

secreting digestive enzymes into the small intestines to help break down food after it leaves the stomach (Busch, 1990). While CF does not directly alter or impair pancreatic function, it causes mucus to clog the organ and prevents the essential enzymes from entering the small intestine. As a result, no matter how nutritious their diet, patients with CF are unable to independently extract essential nutrients from food. As a result, before the advent of enzymatic treatments for CF, patients seldom survived infancy (Andersen, 1938).

The earliest recorded account of a patient dying from what is believed to be CF was by Professor Pieter Pauw in 1595. Pauw was a professor of botany and anatomy in Leiden, The Netherlands. He performed an autopsy on an eleven-year-old girl, who the locals described as "bewitched." The girl appeared malnourished, and when Pauw examined her internal organs, he found that her pancreas was "swollen, hardened, gleaming white" (Quinton, 1999). He attributed this to steatorrhea, a symptom in which abnormal amounts of fats are found in the feces, indicating poor absorption of fat by the intestines. However, it wasn't until 1905 that a physician named Karl Landsteiner showed definitive evidence that this symptom—among others—was characteristic of CF.

There is also evidence that medieval Europeans recognized that unusually salty skin of children with CF. In this era, licking the foreheads of children and infants was a common practice in ritualistic cleaning ceremonies, which were conducted to counteract what were believed to be hexes (Quinton, 1999). If the child's skin tasted salty, "the child was called bewitched or fascinated and was feared to die soon" (Busch et al., 1990). The salty skin of "bewitched" children was recorded across medieval Europe, in what are now 12 different countries (Busch , 1990). The first scientific report of salty skin preceding

the death of a child was written in 1606 by Alonso y de los Ruyzes de Fonteca in Henares, Spain. However, it would take hundreds of years before physicians would realize that pancreatic and dermal symptoms resulted from the same disease.

Medical research concerning CF finally took off in the year 1938, when Dorothy Andersen, M.D., wrote a detailed report outlining the symptoms associated with CF. This report was based on the histories of 49 patients, and encompassed her personal observations and findings, as well as other cases documented in literature and by her colleagues. While pancreatic malfunction was her primary focus, she was the first researcher to correlate it with respiratory and intestinal symptoms (Andersen, 1938). Furthermore, she was able to demonstrate that enzymatic supplements were able to relieve the digestive ailments of her patients (Andersen, 1938). However, it soon became apparent that solving the digestive problems associated with CF was only the first of many hurdles that would challenge researchers in the subsequent decades.

In the years after Dorothy Andersen's comprehensive report, the prognosis of CF patients steadily began to improve. While many digestive symptoms could be treated, children and infants instead died of respiratory complications resulting from abnormally thick mucus in the lungs (di Sant'Agnese et al., 1946). Subsequently, research was diverted to developing techniques to clear the patients' airways to facilitate breathing.

It was not until 1988—just before the explosion in research known as "the human genome project"—that Francis Collins, Lap-Chee Tsui, and John R. Riordan identified the first mutation associated with Cystic Fibrosis. One year later, in 1989, Lap-Chee Tsui and his team of researchers at the Hospital for Sick Children in Toronto discovered the gene that, when mutated, causes CF (Tsui et al., 1991). This is now known as the CFTR

gene, and since its discovery, over 1,700 different mutations have been identified in connection with CF ("Cystic Fibrosis Foundation: What is CF?"; Bobadilla et al., 2002).

Once the CFTR gene was identified, along with over one thousand disease causing mutations, research has now focused on the treatment of respiratory infections of patients with CF. The life expectancy of CF patients has increased dramatically over the past century (Jerry A. Nick, M.D., "Cystic Fibrosis Life Expectancy"). In the late 30's, children with CF hardly survived infancy. By the 1960's, the life expectancy rose to ten years. As antibiotic therapies and airway clearance treatments advanced, the life expectancy of such patients increased to 15 years by 1970. This figure plateaued for about a decade and a half, however, until double lung transplants became a possibility. The first successful long-term double lung transplant on a CF patient was performed by a physician named Joel D. Cooper in 1988 (Pasque et al., 1990). After this, the average CF patient went from surviving until their mid to late teens, to surviving into their late twenties by 1990. Over the subsequent decades, advancements in antibiotic therapies and other techniques have steadily raised the life expectancy of CF patients to 37 years in the United States (Jerry A. Nick, M.D., "Cystic Fibrosis Life Expectancy").

Life with Cystic Fibrosis

Cystic Fibrosis has a profound effect on a patient's life from the very beginning. However, in modern times, the quality of life of CF patients is higher than it has ever

been. The first step to getting treatment for CF is a conclusive diagnosis. The vast majority of patients are diagnosed with CF during infancy or early childhood—typically before age 3. In the United States, roughly 66% of CF patients are diagnosed before their first birthday, and another 6% are diagnosed before their second birthday. In milder cases, some children do not exhibit symptoms of CF until later in childhood. About 20% of CF patients are diagnosed between the ages of 2 and 15. Only about 6% of CF cases are diagnosed after age 16 ("Cystic Fibrosis Foundation: Caring for a Child with CF," 2016).

In order to diagnose CF, doctors perform a sweat test which determines the sodium and chloride levels in the patient's sweat (Gibson et al., 1959). Elevated sodium and chloride levels in the perspiration of the patient, along with other CF symptoms, allows doctors to make a definitive diagnosis. The test is non-invasive, and doesn't involve any injections. The test is begun by applying pilocarpine, an odorless, colorless chemical, to a small patch of skin on the patient's arm to stimulate the sweat glands. A mild, painless electrical current is then applied to the arm for five minutes to further stimulate sweating. The sweat is then collected from with filter paper or gauze over the next 30 minutes. The sample is then sent to the diagnostic lab, where it is analyzed for elevated salt levels.

After a positive diagnosis, children with CF must receive regular medical attention. Due to the complexity of the disease, patients must be treated for a variety of symptoms. CF patients must clear their airways twice daily, and there are a variety of ways that this can be done, though all methods involve some degree of coughing in order to rid the lungs of excess mucus (Flume et al., 2009) Many patients' airway clearance

routine begins with the use bronchodilators—commonly known as inhalers—to widen the bronchi and relax the muscles around the airways. One common method used to clear airways is done by cupping the hand and clapping it on the patient's back and chest. The patient sits in various positions to loosen mucus in different sections of the lungs. This procedure loosens up the mucus in the lungs so that the patient is able to cough it up ("Cystic Fibrosis Foundation: Airway Clearance Techniques," 2016; Bradley et al., 2006). After loosening the mucus and coughing it up, patients then take inhaled medicines through a nebulizer (Donaldson et al., 2006). These medicines include antibiotics in order to prevent bacterial infection.

Like many other conditions, CF patients have to be very cautious about personal hygiene. CF patients are not immunocompromised, but the thick lung mucus leaves them extremely vulnerable to infection. Since these infections are often difficult or even impossible to eradicate using antibiotic therapies, preventing bacterial colonization on the airways is a key step in prolonging life expectancy and increasing quality of life ("Cystic Fibrosis Foundation: Caring for a Child with CF"). Children with CF may still attend school or daycare, but caregivers must be particularly cautious. It is important that CF children wash their hands regularly and avoid contact with individuals that may be sick.

Proper nutrition is also essential to maintain the health of CF patients. Doctors encourage these patients, especially children, to receive high-fat, calorie-rich diets with plenty of vitamins and minerals (Ramsay et al., 1992). However, problems with pancreatic function often prevent the proper absorption of these nutrients, because the enzymes necessary to degrade food in the intestines are often absent or too few in number to work properly. As a result, CF patients must take pancreatic enzyme supplements with

every meal, including snacks, to ensure proper nutrition. Failure to do so can result in malnutrition and stunted growth (Bronstein et al., 1992).

Through advancements in genetic research, it is believed that gene therapy could be used in the future to treat CF patients. Gene therapy would involve inserting correct (non-mutant) copies of the CFTR gene into the epithelial cells of patients (Burney et al., 2012). The cells could then take up the correct genetic information and repair the transmembrane conductance regulator proteins on the cell membranes. The earliest attempt at gene therapy for CF patients occurred in 1993 (Collins et al., 1993), which used viral and non-viral gene transfer agents to insert the genetic information into the cells. This trial was unfortunately unsuccessful, but further research is ongoing into the potential efficacy of gene therapy. One of the main hurdles that researchers must face is that since CF is a lifelong disease, the epithelial cells are continuously being turned over and replaced with new cells—a normal part of everyday life. However, maintaining the corrective genetic sequence as the cells are continuously replaced is a problem that researchers have still not been able to solve.

Although CF patients are living many years longer than they have in the past, chronic infections are still the major concern for modern CF healthcare. While the initial stages of the infection are typically manageable with antibiotic therapy, eventually the treatment's effectiveness will begin to decline. Once antibiotic treatment stops being effective, CF patients must have a double lung transplant (Yankaskas et al., 1998). However, there are far more patients who need new lungs than there are available lungs for transplant (Saidi et al., 2014). Another complication is that CF patients must receive two new lungs—a single lung transplant is not enough. If only one lung is transplanted,

the infection will persist in the original lung and infect the new lung as well (Shennib et al., 1992). Donated lungs are given to patients based on need, so patients who are in the later stages of infection are given priority. Unfortunately, many patients die on the list awaiting transplant, because there are simply not enough donated lungs to accommodate all the patients on the list.

Fortunately, due to advancements in physical and antibiotic therapies, CF patients in the 21st century are able to lead longer, healthier lives. With proper care and management of symptoms, CF patients are able to attend college, get married, and even have children. The fact still remains, however, that the average CF patient in the United States suffers from chronic lung infections. In the later stages, these infections can be debilitating, and the average life expectancy in the United States is only 37 years ("Cystic Fibrosis Foundation: Life with CF," 2016). It is therefore imperative that more effective treatments for these infections be developed to increase the quality of life and life expectancy of these patients.

Background: *Pseudomonas aeruginosa*

What is Pseudomonas aeruginosa?

Pseudomonas aeruginosa is the major bacterial pathogen that affects patients with CF. It is classified as a Gram-negative bacteria, which is a common method of differentiating species of bacteria. Gram staining is performed by adding a crystal violet dye that can stain peptidoglycan—a polymer that surrounds the cell membranes of certain species of bacteria. Gram-negative bacteria are characterized by having an inner cytoplasmic membrane as well as an outer membrane, with a thin layer of peptidoglycan in between. Gram-positive bacteria, on the other hand, lack the outer membrane and instead have a thicker layer of peptidoglycan surrounding the cell. These morphological differences are used for identification of the species, as well as for determining effective treatments in the case of infection (Hucker et al., 1929).

P. aeruginosa is a rod-shaped bacterium typically 0.5 - 0.8 µm by 1.5 - 3.0 µm in size that is motile by means of one or two flagella (Schniederberend et al., 2013). It has a distinct sweet, earthy odor, and can be easily recognized by its often bright blue-green color. This color is the result of pyocyanin, a toxin produced by P. aeruginosa in order to kill other species of bacteria nearby. While it is not highly toxic to macro organisms, there is evidence suggesting that pyocyanin plays a role in the persistence of infections (Lau et al., 2004).

Additionally, *P. aeruginosa* is an opportunistic pathogen, meaning that it can live outside of the human body in addition to causing infection (Govan et al., 2007). In contrast, many bacteria that are well known for causing disease in humans are obligate pathogens. This means that in order to survive they must exist within a host, which causes infection. One example of an obligate pathogen is *Mycobacterium tuberculosis*, the bacteria that causes tuberculosis (Forrelland et al., 2013). Obligate pathogens cannot

survive outside of a host, meaning that they are not typically isolated from environments other than the human body. This means that in order to contract an opportunistic pathogen, a patient must come in contact with an infected individual.

Opportunistic pathogens operate very differently, however, and can be contracted from a wide variety of sources. *Pseudomonas aeruginosa* is also an opportunist, though it is not known to frequently infect healthy individuals. Instead, *P. aeruginosa* typically infects vulnerable people, such as patients with weakened immune systems, open wounds, or CF (Friedrich et al., 2016). In addition, *P. aeruginosa* is also known to infect a wide range of organisms, including plants and animals (Emerson et al., 2002; Rahme et al., 1995).

One distinguishing factor of *P. aeruginosa* is its relatively large genome size. *P. aeruginosa* has a genome size between 5.8-6.8 million base pairs and over 5,000 genes (Stover et al., 2000). For comparison, *E. coli* has about 4.5 million base pairs and 4,000 genes. The large genome size of *P. aeruginosa* offers some explanation regarding the astounding variety of stimuli that the bacterium is able to respond to. This can be clearly demonstrated by the sheer number of environmental niches that *P. aeruginosa* can occupy. This organism has been isolated from soil, water, plants, humans, insects and other invertebrates (Corby-Harris et al., 2006; Emerson et al., 2002; Rahme et al., 1995). It is also very tolerant to a wide temperature gradient, and exhibits normal growth between 25 and 42 degrees Celsius (Tsuji et al., 1982).

Biofilm formation is another hallmark of *P. aeruginosa*, which can be observed in both infections and in nature. A biofilm is formed by clusters of cells that stick together and adhere to a surface. Biofilms containing *P. aeruginosa* can be found in many

different habitats, such as plant roots, the human body, and household plumbing (Walker et al., 2003; Moritz et al., 2010).This group of cells is typically surrounded by extracellular polymeric substance (EPS), which gives biofilms their characteristic slimy appearance. Cells in biofilms behave remarkably different from planktonic (free-living) cells both phenotypically and physically, for example, biofilms can exist as complex three dimensional structure in response to a variety of environmental factors.

Biofilm formation is also crucial for another behavior that this species exhibits. *P. aeruginosa* can form biofilms of varying sizes, including microscopic aggregates and biofilms that are visible to the naked eye (Rasamiravaka et al., 2015). When the cell density of *P. aeruginosa* aggregates reaches a certain threshold, the species is known to utilize a form of cell-to-cell communication known as quorum sensing (QS) (Wagner & Iglewski, 2008). This is an extremely basic form of communication used by some bacteria and insects in order to coordinate group behavior to benefit the survival of the community. Organisms with QS ability release signaling molecules in response to certain stimuli, which are received and interpreted by other individuals in the vicinity. It is known that at certain population density levels, *P. aeruginosa* uses QS to coordinate the upregulation or downregulation of certain genes throughout the local population. The regulation of these genes results in coordinated group responses to potential threats, which helps the population to persist (Whiteley et al., 2001; Wagner & Iglewski, 2008).

Pseudomonas aeruginosa Pathogenicity

In addition to being able to occupy a wide array of niches, *P. aeruginosa* is also known for its ability to cause infections in a variety of organisms. Plants, invertebrates, and other animals—including humans—are all potential targets (Corby-Harris et al., 2006; Emerson et al., 2002; Rahme et al., 1995). *P. aeruginosa* has also shown a striking ability to retaliate its host's defense systems by altering its spatial organization and gene regulation (Wagner & Iglewski, 2008; Rasamiravaka et al., 2015). While *P. aeruginosa* is not particularly virulent towards healthy organisms, once it secures a foothold within a host, it can be almost impossible to eradicate (Al-Aloul et al., 2004; Hart & Winstanley, 2002).

Studies have shown that under certain conditions, *P. aeruginosa* occupying soil habitats are able to infect plants by colonizing the roots. A study conducted in 2004 showed that *P. aeruginosa* is able to infect Arabidopsis—a model organism used by plant biologists—and sweet basil both *in vivo* and *in vitro* (Walker et al., 2003). In response to the contact with pathogenic *P. aeruginosa*, basil plants secrete antibacterial compounds from their roots. Although the secretions are toxic to planktonic cells, the study found that *P. aeruginosa* was almost completely unaffected by this compound. Instead of being killed off by the toxin, *P. aeruginosa* responded by reverting from its planktonic form into a biofilm before the concentrations of the toxins were able to reach lethal levels. The basil plant's attacks were unable to penetrate this biofilm, and both plants—basil and Arabidopsis—succumbed to the infection.

While plant infections do not directly relate to human health, the observations from the aforementioned experiment are somewhat analogous to how *P. aeruginosa*

behaves in human infections. *P. aeruginosa* is notorious for infecting humans in handful of specific circumstances. It has been known to colonize the urinary tract, airways, wounds, and medical devices (Pavlovskis & Wretlind, 1979; Bodey et al., 1983). Of these types of infections, the most common—and most problematic—are chronic infections of burn wounds and the lungs of CF patients.

Chronic bacterial lung infection is by far the leading cause of mortality among CF patients, and *P. aeruginosa* is one of the mostly isolated pathogens. It is estimated that between 70-80% of adults with CF have chronic lung infections, and 98% of these patients will be colonized with *P. aeruginosa* within 3 years of the initial infection (Govan et al., 2007). Compared to other pathogens, *P. aeruginosa* grows fairly slowly, so patients may not experience symptoms for some time after the initial colonization. In fact, most adults who test positive for the presence of *P. aeruginosa* in their lungs are believed to have initially contracted the bacteria in their childhood or teens (Gibson et al., 2003).

At the early stages of *P. aeruginosa* infection, CF patients are typically colonized by a number of different strains, though over the course of the infection, one strain tends to dominate (Govan et al., 2007). However, these strains do not remain unchanged over time. *P. aeruginosa* is known for its ability to mutate readily in order to adapt to stressful environmental factors, including antibiotics. Unlike many other bacterial species, *P. aeruginosa* does not need to rely on spontaneous mutations to alter its genome. Instead, *P. aeruginosa* is able to undergo recombination, the process by which individuals are able to exchange DNA. For most macro organisms—which usually have two parents—recombination is used to shuffle parental genes in order to produce genetically unique

progeny. Asexual organisms, by contrast, typically produce offspring that are genetically identical to their parents. Therefore, in order to evolve, many of these organisms must wait for a beneficial mutation to spontaneously arise. In *P. aeruginosa*, however, recombination is the driving force for diversity (Darch et al., 2015), allowing it to evolve rapidly and efficiently.

The highly mutable nature of *P. aeruginosa* has very serious implications for hospitals caring for CF patients. The ubiquitous nature of the organism means that it can be inadvertently spread between hospital patients through many vectors, including water, surfaces, and medical instruments. Since individuals tend to harbor unique, often highly resistant strains of *P. aeruginosa*, transmission of these strains between CF patients can have disastrous consequences. One example of such an event is the spread of the "Liverpool Epidemic Strain" in a children's CF clinic in the mid 1990's (Cheng et al., 1996). The strain was resistant to ceftazidime, azlocillin, and imipenem, and was shown to be present in 55 of the 65 children at the clinic.

CF patients can contract *P. aeruginosa* in a number of ways, due to the ubiquitous nature of the organism. In most cases, *P. aeruginosa* is believed to be contracted from the environment. *P. aeruginosa* can grow in two different forms—mucoid and colonial—and each form has different implications for the patient. During early colonization, *P. aeruginosa* typically grows in colonial form, meaning that bacterial colonies resemble circular pinpricks, and look "dry" in appearance (Burns et al., 2001). This form tends to be fairly susceptible to antibiotics (Parviz et al., 2014) so with immediate, aggressive treatment, it is possible to eradicate from the lungs (Frederiksen et al., 1997). This immediacy of this treatment cannot be overstated, as *P. aeruginosa* can rapidly change its

phenotype in the presence of antibiotics (Oliver et al., 2000). This is very similar to the aforementioned experiment involving basil plants. While the plants are capable of producing enough of the defensive toxin to completely kill planktonic PA, this process does not happen fast enough to kill the bacteria before they develop resistance (Walker et al., 2003). Therefore, appropriate antibiotics must be administered to infected CF patients quickly and at a high enough concentration to completely kill the pathogen. However, patients often do not exhibit symptoms when the bacteria are in this stage of growth, so the presence of *P. aeruginosa* is typically not detected until it has developed the mucoid phenotype .

As the name suggests, the mucoid phenotype indicates a mucus-like appearance of bacterial growth. Mucoid growth tends to be irregularly shaped and appear wetter than colonial growth. The mucoid phenotype is associated with a heightened ability to form biofilms (Kenna et al., 2007). Biofilm formation is beneficial to *P. aeruginosa* not only because it makes bacteria less susceptible to antibiotics, but also because the high density of cells facilitates quorum sensing, which allows the bacteria to respond to changes in the environment collectively (Wagner & Iglewski, 2008).

Diagnostic Techniques

When a CF patient exhibits symptoms consistent with bacterial lung infection, it is imperative that he or she get immediate treatment to prevent chronic infection. In order to determine the ideal treatment, doctors must first determine which pathogen is responsible and which antibiotic to prescribe. Selecting the most effective antibiotic is especially

difficult if the pathogen is determined as *P. aeruginosa*, because different strains of this species can have extremely varied responses to different antibiotics (Kenna et al., 2007).

In order to select the appropriate antibiotic for each patients, diagnostic labs perform a test known as a disk diffusion assay (Bauer, 1959; Bauer & Kirby et al., 1966). The first step of this assay is to obtain a sample of lung mucus (formally referred to as "sputum") from the patient. The patient—using aforementioned techniques—coughs up infected sputum, which is collected and sent to the lab. There, the sample is analyzed to determine which species are present. This is done in a variety of ways, such as monitoring growth rate, observing distinctive morphological and metabolic characteristics, and using selective media to isolate specific species (Washington, Chapter 10). Due to its distinctive sweet, earthy smell and blue-green coloration, *P. aeruginosa* is not difficult to identify. Additionally, because of its prevalence among CF patients, diagnosticians frequently look for its presence in the lung sputum.

Once *P. aeruginosa* is isolated from the sample, the bacteria are grown in a Petri dish on nutritionally rich, non-selective media (Bauer, 1959; Bauer & Kirby et al., 1966). MHA is a common media used by diagnostic labs to test *P. aeruginosa*, as it is highly rich in nutrients and facilitates rapid bacterial growth. The bacteria are swabbed over the entirety of the agar surface, creating what is called a "bacterial lawn." Afterwards, the lab technicians apply paper disks soaked with different antibiotics to the Petri dish. The disks—typically only one centimeter in diameter—are spaced apart from each other so that no cells are exposed to more than one antibiotic at a time. The antibiotics from these disks then diffuse into the agar over night as the bacteria grow. If the strain of *P. aeruginosa* is susceptible to the antibiotic, a zone of inhibition—areas that lack bacterial

growth—will be visible on the plate surrounding the antibiotic. The diameter of this kill zone directly relates to how susceptible the strain is to the antibiotic. From this test, doctors are able to determine what is known as the antimicrobial susceptibility profile (the level of susceptibility or resistance of the strain to different antimicrobial agents) of each patient's particular strain of *P. aeruginosa*.

While this assay works well for many pathogens, this is unfortunately seldom the case for *P. aeruginosa*. Doctors may request that this test be done for the sake of following procedure, but most are aware that the susceptibility profiles produces are typically not indicative of how a patient will respond to the treatment. Instead, doctors often rely on their past experiences in treating *P. aeruginosa*, and prescribe antibiotics according to what they know about the species. Doctors also look at antibiotics that their patient has taken in the past, which can indicate what the strain has previously been exposed to—and therefore— competitors—and grows under relatively little stress. Furthermore, the media used by diagnostic labs is what it may have developed resistance to.

When one takes into account the plasticity and adaptability of *P. aeruginosa*, it becomes clear why this test produces such erroneous results. This species is known for its ability to thrive in a wide range of environments, which can be attributed, in part, to its relatively large genome (Stover et al., 2000; Corby-Harris et al., 2006; Emerson et al., 2002; Rahme et al., 1995). When *P. aeruginosa* encounters a new habitat, it is able to change its behavior accordingly through both mutation and altered gene expression. The nutritionally rich media used by diagnostic labs does not resemble the conditions of a human CF lung in any way. On the rich media, *P. aeruginosa* has few obstacles to adjust

to—such as host immune response and bacterial competitors—and grows under relatively little stress. Furthermore, the media used by diagnostic labs is more nutritious than CF lung sputum, which further alters the behavior of the bacteria. Although the strain may exhibit sensitivity to an antibiotic in this *in vitro* model, this behavior cannot be correlated to how it will behave inside of a human lung. This is the microscopic equivalent of trying to learn about orca whale hunting techniques by observing orcas at sea world, or trying to learn about lion behavior by observing one in a hotel room. In order to get an accurate picture of an organism, the *in vitro* model must recapitulate the *in vivo* as closely as possible.

It is undeniable that prescribing the correct antibiotics to a CF patient is critical for preventing chronic infection and prolonging life expectancy. As such, making improvements to diagnostic testing of *P. aeruginosa* is absolutely vital. In my thesis, I propose that a modifications to the current diagnostic method in order to more closely emulate the *in vivo* in a clinical setting. In my experiments, I compared the responses of 10 different strains of *P. aeruginosa* in two different types of growth media. The first media, Mueller-Hinton, is a rich media commonly used in diagnostic labs to test obtain the antimicrobial susceptibility profile of *P. aeruginosa.* The second media is called Synthetic Cystic Fibrosis Media 2 (SCFM2), which is a chemically defined media that has similar physical and nutritional properties to CF lung sputum.

These strains of *P. aeruginosa* were analyzed using minimum inhibitory concentration assays (MIC) in liquid media and disk diffusion assays on solid media. These assays were performed in the artificial sputum media and in the MH media. The data from these experiments was analyzed to generate antimicrobial susceptibility

profiles of each strain in each type of media, in order to see if the cells responded to antibiotics differently depending on the environment in which they are grown. The results indicated that all ten strains of *P. aeruginosa* exhibited varying levels of resistance and susceptibility to three clinically relevant antibiotics depending on the medium the cells were tested in.

Section Two: Materials and Methods

MIC Light Assay Protocol

The minimum inhibitory concentration assay (MIC) was performed using a 96 well plate, Luminoskan Ascent microplate luminometer, and 3 clinically relevant antibiotics: polymyxin B, tobramycin sulfate, and gentamicin. Antibiotics were dissolved in nanopure water, filter sterilized, and stored, covered with aluminum foil, at 4°C. The selected medium—SCFM2 or Mueller-Hinton Broth (MHB) was then inoculated with *P. aeruginosa* strains carrying the luminescence reporter plasmid pQF50- lux (Murray et al., 2015) standardized to an OD_{600} of 0.05 after washing cells twice in phosphate buffered saline (PBS). 200μL of inoculated media was added to the wells in the third column, and 100μL of sterile media was added to the first column as positive and negative controls respectively. The remainder of the wells were filled with 100 μL of inoculated media. Antibiotics were added to the wells third column and then serially diluted two-fold across the wells, producing an antibiotic concentration gradient ranging from 2.5mg/ml to 0.005mg/ml. The 96 well plate was then inserted into the Luminoskan Ascent microplate luminometer. The luminometer measured luminescence at 0 minutes, 30 minutes, and 60 minutes from each of the 96 wells.

For this assay, luminescence is a proxy for cell viability. The levels of luminescence for each well treated with antibiotics were compared to wells that did not receive antibiotics. Results are presented as a percentage of cell viability for each well. In other words, a percentage of the bacterial population that survived the antibiotic. This assay was replicated three times for each strain.

Disk Diffusion Protocol

The disk diffusion assays were performed using agar plates and antibiotic disks. These disks are circular pieces of paper, 1 cm in diameter, that contain 10 micrograms of antibiotic. The antibiotics used in this assay were tobramycin, gentamicin, and colistin sulfate (also known as polymyxin E). All agar plates contained 15ml of either SCFM2 or MHB agar. Prior to adding bacteria, plates were dried for 30 minutes in a biological cabinet. The day before the assay, bacterial strains to be tested were isolated onto LB agar plates, wrapped in aluminum foil, and allowed to grow overnight at 37°C.

On the day the assay is performed, 3 distinct colonies from each strain were selected. Each colony was inoculated into 1ml of sterile, nanopure water. The inoculated water was then streaked across the dry agar plates using a sterile cotton swab, ensuring that the entire surface of the agar is coated. Using small tweezers, one disk of each antibiotic was added to each plate (tweezers are rinsed with 70% ethanol and passed through a flame after each placement to prevent contamination). Plates were then inverted, wrapped in aluminum foil, and allowed to grow overnight at 37°C. Exactly 24 hours after the disks were applied, the diameter of the zones of inhibition around each antibiotic disk were measured. This assay was replicated three times for each individual strain.

Media Construction, Design and Composition

<u>Mueller-Hinton broth, Muller-Hinton agar</u>

Mueller-Hinton media is a rich, general purpose bacterial growth media. The brand used in these experiments was BD (Becton, Dickinson and Company) brand. The formula

contains 3.0g beef extract, 17.5g acid hydrolysate of casein, and 1.5g of starch per liter. For solid Mueller-Hinton media, agar was added at a concentration of 1% weight per volume. Media is autoclaved at 121°C at a pressure of 15psi for 20 minutes. 15mL of solid Mueller-Hinton media is added to Petri dishes by pipetting. Media is stored at 4°C.

Synthetic cystic fibrosis sputum media (SCFM2)

SCFM2 is comprised of four main components: A buffered base containing essential amino acids and salts, DNA, mucin, and the lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

DNA purification

The DNA in this medium is derived from salmon sperm, and must be purified before being added to the media. 250 milligrams of dehydrated salmon sperm is dissolved in di-water at a concentration of 1 milligram of DNA per 1 milliliter of di-water. It takes roughly two hours for the DNA to completely dissolved. The solution is aliquoted into 50mL conicals. An equal volume of cold (4°C) phenol-chloroform (PCL) is added to each conical. The conicals are then placed into a centrifuge that has been cooled to 4°C. The tubes are spun at 5000xg for 10 minutes. The solution will separate into 2 phases. The top phase is pipetted out and transferred to a fresh conical. 0.1 volumes of NaOAc at pH 5.2 is then added to each conical. 2 volumes of cold 100% ethanol are added to each conical. The conicals are then covered with aluminum foil and stored at -20°C for a minimum of 2 hours.

After incubation, the conicals are centrifuged at 4°C at 5000xg for 30 minutes. The DNA will pellet at the bottom on the conical. The supernatant is then removed from the conical and 1 volume of cold 70% is added. The solutions are placed back into the

4°C centrifuge at 5000xg for 10 minutes. The supernatant is removed, and another volume of cold 70% ethanol is added. The solutions are centrifuged at 4°C at 5000xg for 10 minutes. The supernatant is removed again, and the tubes are placed, uncovered, in a chemical hood to dry overnight. Once the pellets are completely dry, the pellets are removed from the conicals with tweezers and placed in Petri dishes. The pellets are weighed, and then placed, uncovered, in a UV sterilization drawer for 30 minutes. The Petri dishes are then removed, wrapped in parafilm and aluminum foil, and stored at -20°C.

Porcine Mucin

250mg of dehydrated mucin is weighed out into Petri dishes and placed, uncovered, into the UV sterilization drawer. The mucin is sterilized for 4 hours, mixing once every 30 minutes. Mixing is done by replacing the lid and gently shaking the Petri dish. The lid is then removed again and left in the UV drawer. Mucin is then stored at -20°C for future use.

Buffered Base

There are 34 individual liquid stocks that are combined when making the buffered base. These stocks include but are not limited to 19 amino acids, salts, and MOPS. Stocks are made by dissolving solids in nanopure water and then filtering sterilizing. All stocks are stored at 4°C, covered with aluminum foil, for up to two weeks. Appropriate amounts of the stocks are then added to 190mL of nanopure water, mixed, and filtered sterilized.

SCFM2 Construction

The media is made by combining appropriate amounts of mucin, DNA, and the buffered base. Upon addition of sterilized DNA and mucin, the lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) is added at a concentration of 100ug/mL. DOPC is first dissolved in chloroform at a concentration of 250mg/ml, however, in order to allow it to better dissolve into the buffered base. The media is then placed in an incubator at 37°C for one hour, shaking at 250rpm to evaporate chloroform. The SCFM2 media is stored at 4°C for up to two weeks.

SCFM 2 agar

The solid version of the SCFM2 media is prepared in a similar fashion to the liquid media. However, the buffered base is made twice as concentrated (2 times the amount of stocks added to the original volume of nanopure water). Twice the amount of DNA and mucin is also added. In another flask, nanopure water is added at a volume equal to the concentrated buffered base. Agar is added to the nanopure water at a concentration of 2% of the total volume of water, which is equivalent to 1% of the buffered base and water when combined. The agar solution is well mixed and autoclaved at 121°C at a pressure of 15psi for 20 minutes to sterilize.

Once the chloroform from the added DOPC solution has been evaporated from the buffered base and the agar solution has been autoclaved, both flasks are placed into a 50°C water bath for one hour, or until the temperatures of both solutions are equivalent. Working aseptically, the buffered base is pipetted into the agar solution 50mL at a time. Using the stir bar that remained in the agar flask in the autoclave, the solutions are mixed together for 5 minutes, or until the solution is homogenous. The solution is pipetted into Petri dishes in 15mL aliquots.

Strains: Origins and Significance

PAO1

PAO1 is the most common strain of *Pseudomonas aeruginosa* used in laboratory research that exhibits the colonial (non-mucoid) phenotype. It is a wild-type strain of *P. aeruginosa*, meaning that its genotype is the natural (non-mutant) form of the species. Because of this, PAO1 is commonly used as a control—or "reference"—strain. It is derived from an initial isolate called PAO. This strain was isolated from a patient in Australia by B.W. Holloway in 1955. Since then, the strain and its variants have been distributed to labs worldwide to be used in experimental research (Klockgether et al., 2010). The strain of PAO1 used in these experiments carried plasmid pQF50-lux. This gene on this plasmid contains a luminescence expressing gene, with causes living cells to produce light.

PA14

This strain of *P. aeruginosa* is another common strain used in laboratory research that, like PAO1, has the colonial phenotype. PA14 is considered more virulent than PAO1, as it carries pathogenicity islands not present in the *P. aeruginosa* core wild-type genome (He et al., 2004). While this research project is only concerned with impacts on human health, it is worth noting that PA14 is able to colonize both plants and animals. In these experiments, PA14 carried plasmid pQF50-lux, which causes living cells to produce light.

SED4, SED6, SED9, SED20, SED21

These five strains are clinical isolates, as opposed to lab or wild-type strains. These isolates were provided in collaboration with Nottingham University Hospitals NHS trust. The samples were isolated from sputum samples obtained from a 19 year old female with CF, who had been chronically infected with *P. aeruginosa* for 3 years. At the time when the samples were collected, the patient was in stable condition and was prescribed maintenance oral and nebulized therapies (Darch et al., 2015). The morphological characteristics between these strains are relatively consistent—all produce observable amounts of pyocyanin and grow in colonies. These strains were selected for this study via a random number generator.

C2773C, C1913C, C3881C

These 3 strains are clinical isolates collected from 3 different CF patients. These patients were at British Columbia's Children's Hospital, Shaughnessy Hospital, or St. Paul's Hospital in Vancouver, British Columbia, Canada. The isolates were obtained from either lung sputum or throat samples (Huse et al., 2010). Unlike the other set of clinical isolates, these strains were isolated from separate patients, are mucoid, and produce lower levels of pyocyanin in comparison. These strains were selected for this study via a random number generator.

Plasmid Transformation of Clinical Strains

In order to perform minimum inhibitory concentration assays (MIC) on these strains, all were transformed with plasmid pQF50-lux via electroporation. To do this, cells were first grown in 5mL of LB at 37°C shaking at 250rpm. These strains have longer doubling times than PAO1 and PA14, therefore cells were grown for 36-48 hours. Cells were then removed from the incubator, and 200mL of each culture were added to 5mL of fresh LB

media. Cells were grown in the incubator for an additional 1.5 hours at 42°C, so that cells could reach the exponential stage of growth.

Cells were then removed from the shaker and placed on ice for 10 minutes. All cultures were then transferred to 15mL conicals and spun in a 4°C centrifuge at 5000xg for 4 minutes. Since the cells then form pellets at the bottom of the tubes, the media can then be poured out and disposed of. To wash the cells, 5mL of cold 300mM sucrose solution was added to each culture, and pipetted until the cells were resuspended. Cells were spun in the 4°C centrifuge at 500xg for 4 minutes, and the supernatant was poured off. Cells were washed 2-3 times. After pouring off the supernatant a final time, 50 μL of cold 300mM sucrose solution was added to each conical. 5 μL of the plasmid DNA is then added to each strain. The cells were pipetted until resuspended, transferred to chilled cuvettes, and then placed into the electroporator. A electrical current of 2500V is applied, which causes the bacteria to uptake the plasmid. 1mL of fresh LB media is added into the cuvette, mixed thoroughly, and transferred into a fresh Eppendorf tube. Strains are then placed into the incubator at 37°C shaking at 250rpm. Cells are allowed to grow for at least 1 hour.

After incubation, the strains are removed from the incubator and transferred to LB agar plates containing the antibiotic carbenicillin. In addition to the *lux* gene, this plasmid also contains a carbenicillin resistance gene, meaning that cells that correctly took up the plasmid will also be resistant to this antibiotic. Cultures are inoculated onto these plates, wrapped in aluminum foil, and incubated statically at 37°C for 36-48 hours. Cells that grow on the antibiotic-LB plates are then examined under UV light so that luminescence,

if present, can be seen. Cells can also be inserted into the Luminoskan Ascent microplate luminometer—which measures light—to check for luminescence.

If luminescence is detected, a single colony is then selected from the agar plate and transferred to 5mL of LB media along with carbenicillin at a concentration of 300μg/mL. Cells are then grown for 36-48 at 37°C shaking at 250rpm. From these cultures, 50% glycerol stocks of each strain are prepared and stored at -80°C.

Section Three: Results

PAO1 and PA14 exhibit increased resistance to antimicrobials in synthetic CF sputum medium than in standard diagnostic laboratory medium

Results from minimum inhibitory concentration (MIC) assays revealed that PAO1 and PA14 were significantly more resistant to antibiotics when cultured in synthetic CF sputum media. This was consistent for all three time points (0, 30, and 60 minutes) and all three antibiotics (tobramycin, gentamicin, and polymyxin B).

Both PAO1 and PA14 were most susceptible to polymyxin B. It is currently the last line drug prescribed to patients infected with *Pseudomonas aeruginosa*, meaning that it is only administered after all other treatments have failed. Doctors do this partly in order to prevent the bacteria from developing resistance to the antibiotic, however this is not the only reason. Though it is extremely effective and fast-acting against the bacteria, it is also quite toxic to the human patient.

On average, at the lowest concentration of antibiotic (4.88e-4 μg/mL), the number of viable cells of PAO1 grown in MHB was reduced to about 52.6% after 60 minutes (Figure 1). By contrast, PAO1 grown in SCFM2 subjected to the same concentration of polymyxin B showed no significant reduction. For a similar killing of PAO1 in the SCFM2, the concentration of this antibiotic must be increased eightfold. To reduce the bacterial load significantly of PAO1 by the 60 minute time point, a higher concentration of polymyxin B is necessary for cells grown in SCFM2 than in MHB. The concentration for almost complete cell death in MHB was 0.0078μg/mL at the latest time point, compared to 0.0313μg/mL in SCFM2.

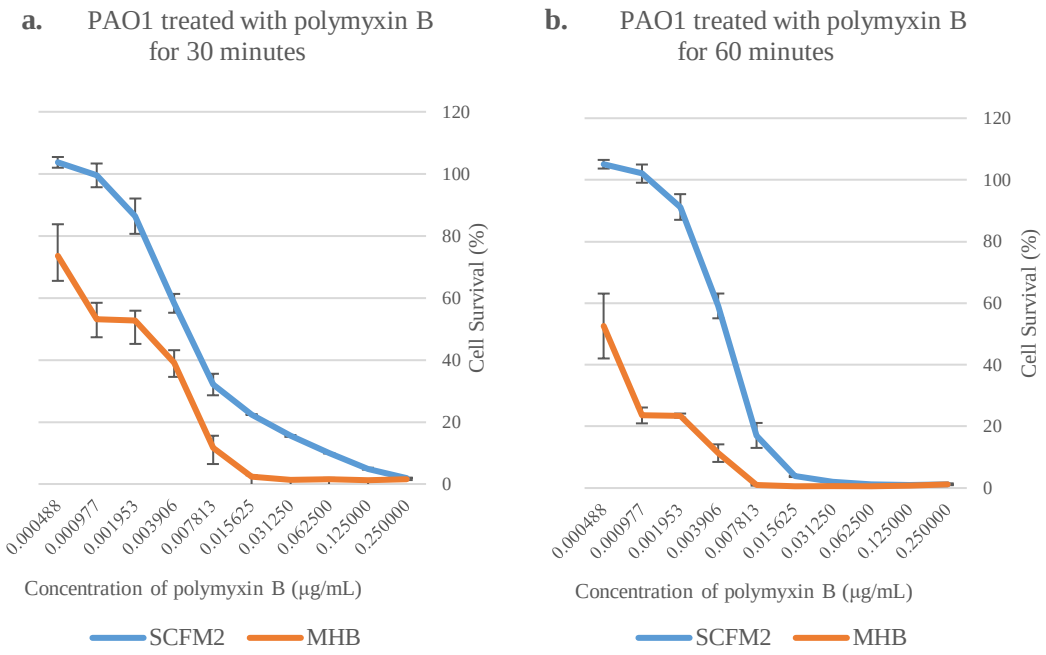
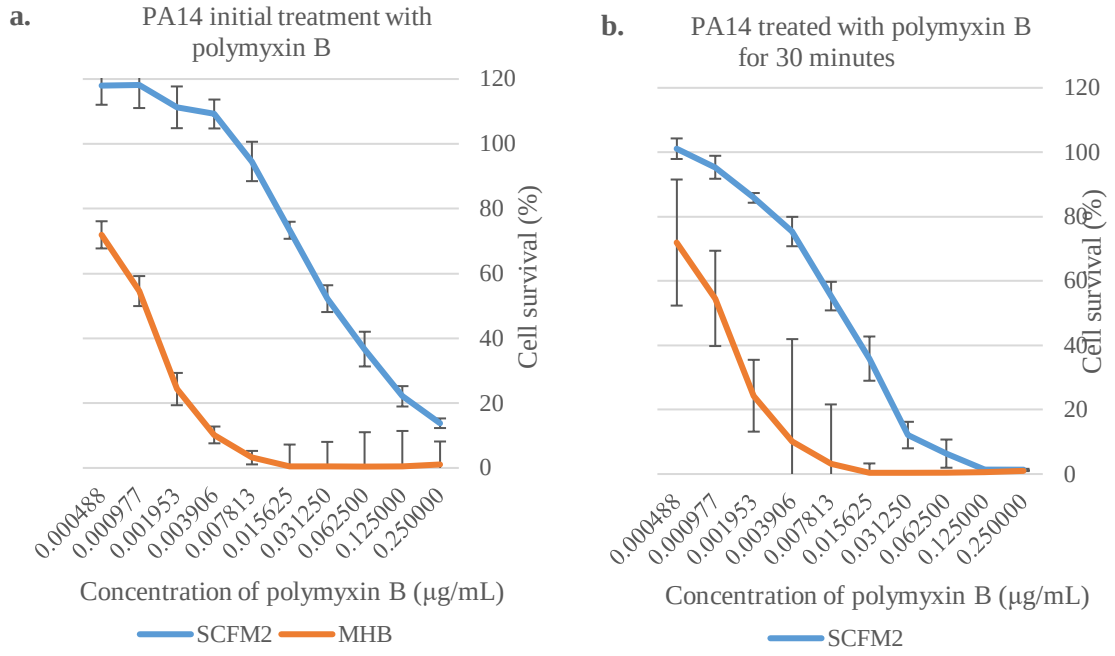


Figure 1 | PAO1 treated with polymyxin B exhibited higher levels of resistance in SCFM2 than in MHB. Increasing concentrations of Polymyxin B significantly impacted effected PAO1 growth in MHB compared to cells grown in SCFM2. At 60 minutes, differences in the percentages of remaining viable cells were significant at concentrations 3.91e-3µg/mL ($p < 0.0001$), 1.95e-3µg/mL ($p < 0.0001$), 9.77e-4 µg/mL ($p < 0.0001$), and 4.88e-4µg/mL ($p < 0.0001$) as determined by an unpaired t-test. Error bars represent standard error of the mean. (n=3)

PA14 was also susceptible to polymyxin B. In MHB, the population of PA14 was nearly eradicated at the initial time point at an antibiotic concentration of 0.0078µg/mL. By contrast, when PA14 was grown in SCFM2, the same concentration of polymyxin B only reduced the population by about 5%. At the initial time point, even the highest concentration of this antibiotic was not able to eradicate the population when it was grown in SCFM2. Nearly complete killing of PA14 by polymyxin B was achieved in SCFM2 after 30 minutes at a concentration of 0.125µg/mL, the second highest concentration that was used. By contrast, PA14 grown in MHB was almost completely

reduced at the 30 minute time point at a concentration of 0.0156μg/mL, 3 times lower than was necessary in SCFM2. After 60 minutes, the lowest concentration of polymyxin B used was able to reduce the number of viable cells grown in MH by 70%. When subjected to the same concentration of polymyxin B, cells grown in SCFM2 experienced no significant killing.



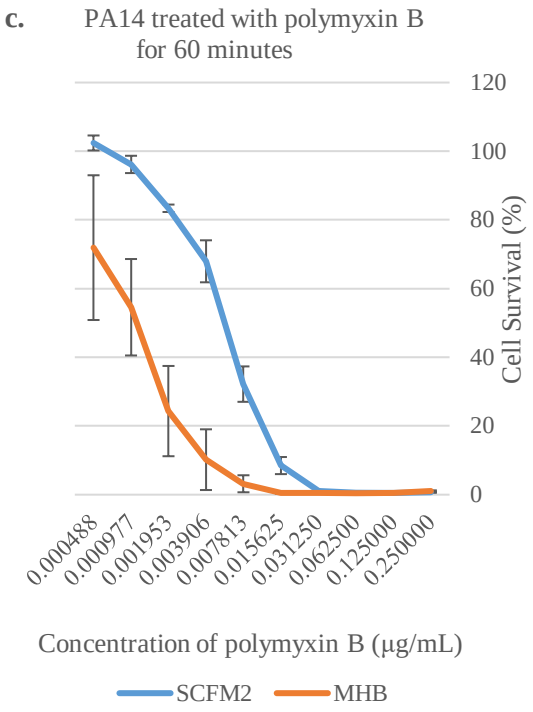


Fig 2 | PA14 treated with polymyxin B was significantly more resistant in SCFM2 than in MHB. a-c. At the initial time point (**a**), differences in cell viability at concentrations 3.91e-3µg/mL to 4.88e-4 were significant as demonstrated by an unpaired t test ($p < 0.0001$). At the 60 minute time point (**c**), differences in cell viability at concentrations 3.91e-3µg/mL ($p= 0.0058$), 1.95e-3µg/mL ($p= 0.0110$), and 9.77e-4µg/mL ($p= 0.0435$) were significant as demonstrated by an unpaired t test. Error bars represent standard error of the mean. (n=3)

Both strains were also exposed to tobramycin. Overall, tobramycin killed that bacteria much more slowly than polymyxin B, and the speed of killing varied depending on the media in which the bacteria were grown. For instance, when the population of PA14 that was grown in MHB was exposed to a tobramycin concentration of 0.125µg/mL, it was reduced to 50.898% of its original size after 30 minutes. By contrast, when exposed to the same concentration of tobramycin, the population of cells grown in SCFM2 was only reduced by 9.582% after 30 minutes. Complete killing of PA14 via tobramycin did not occur at any concentration of tobramycin in either media, although PA14 was significantly more resistant in SCFM2 than in MHB. At the 60 minute time point, PA14 grown in MH media was reduced to 45.6171% of its original population size when exposed to a tobramycin concentration of 0.0156µg/mL. A similar amount of killing (population reduction of 48.0873% compared to original size) in SCFM2 was

achieved at 60 minutes at a tobramycin concentration of 0.06250µg/mL, four times higher than was necessary in MH media.

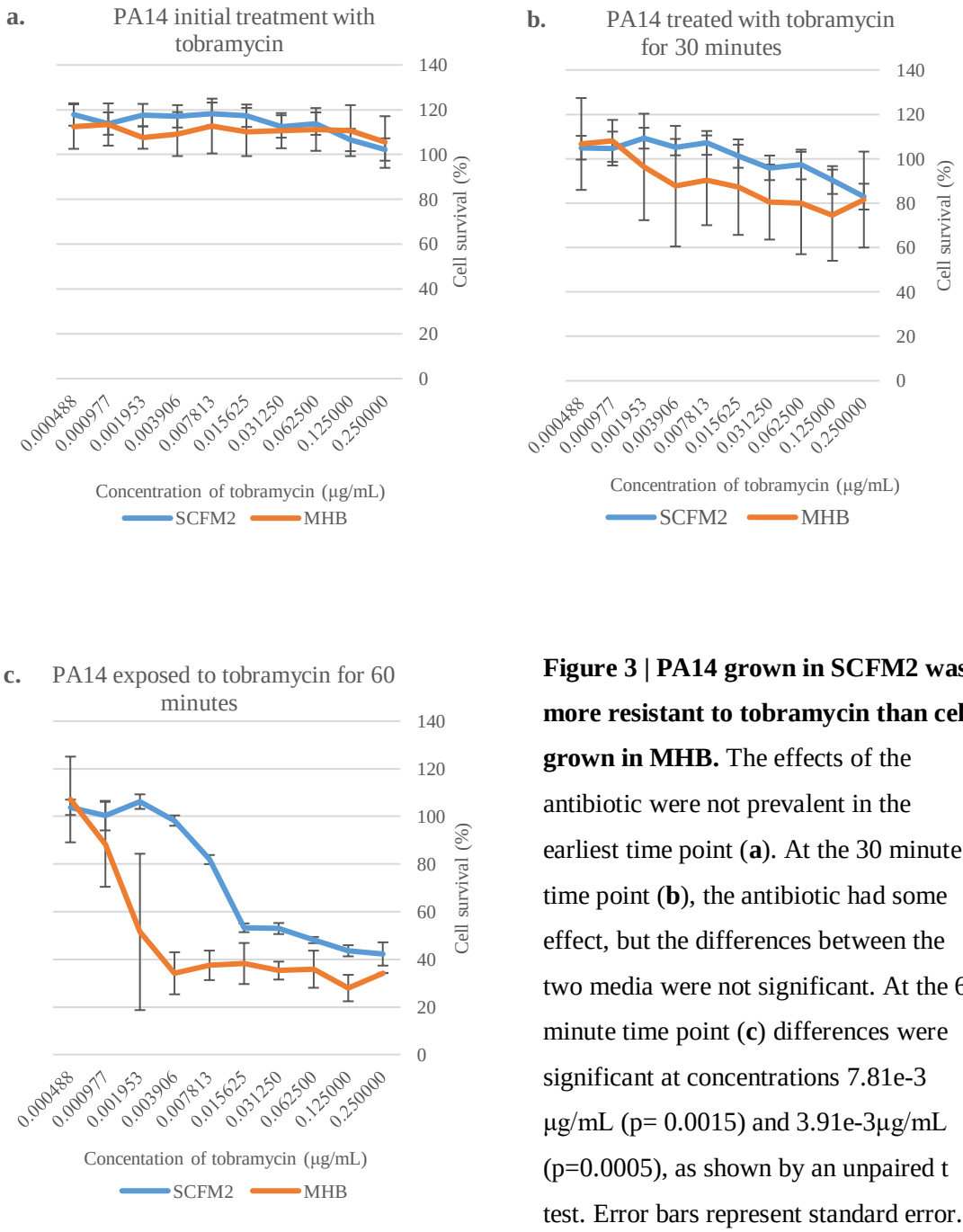


Figure 3 | PA14 grown in SCFM2 was more resistant to tobramycin than cells grown in MHB. The effects of the antibiotic were not prevalent in the earliest time point (**a**). At the 30 minute time point (**b**), the antibiotic had some effect, but the differences between the two media were not significant. At the 60 minute time point (**c**) differences were significant at concentrations 7.81e-3 µg/mL (p= 0.0015) and 3.91e-3µg/mL (p=0.0005), as shown by an unpaired t test. Error bars represent standard error. (n=3)

Laboratory and clinical strains of P. aeruginosa exhibit different antibiotic susceptibility profiles dependent on the type of solid media used

Disk diffusion assays on Muller-Hinton agar (MHA) and SCFM2 agar were conducted using 10 different strains of *P. aeruginosa* and three clinically relevant antibiotics, tobramycin, gentamicin, and polymyxin B. The results from this assay indicated that all strains had differing susceptibility profiles when tested on different media. While some of the strains were more resistant to all antibiotics on SCFM2 agar, this was not always the case.

The disk diffusion assays for PAO1 and PA14 yielded analogous results to the MIC assays conducted in liquid media. For tobramycin, the zone of inhibition for PAO1 was 0.67cm greater in diameter in MHA than it was in SCFM2 agar. Via the percent increase formula, the diameter of the zone of inhibition was 27.92% greater in the Muller-Hinton media than in the SCFM2 agar. Gentamicin produced a zone of inhibition that was 0.87cm greater in MHA than in SCFM2 agar. The diameter was thus 45.79% greater in MHA than in SCFM2 agar. Polymyxin B yielded similar results, with a diameter that was 1.00cm larger and 20.00% greater in MHA than in SCFM2 agar.

PA14 was less resistant to tobramycin and gentamicin than PAO1, though both strains showed similar levels of susceptibility to polymyxin B. Like PAO1, PA14 showed increased levels of resistance—demonstrated by a smaller zone of inhibition—on SCFM2 agar than on MHA. When tobramycin disks were applied, the zone of inhibition was 0.70cm wider and 23.33% greater on MHA than on SCFM2 agar. Gentamicin produced a kill zone that was 0.67cm wider and 24.10% greater on MHA than on SCFM2 agar. For

polymyxin B, the kill zone in MHA was 1.31cm wider and 150.57% greater than it was in SCFM2 agar.

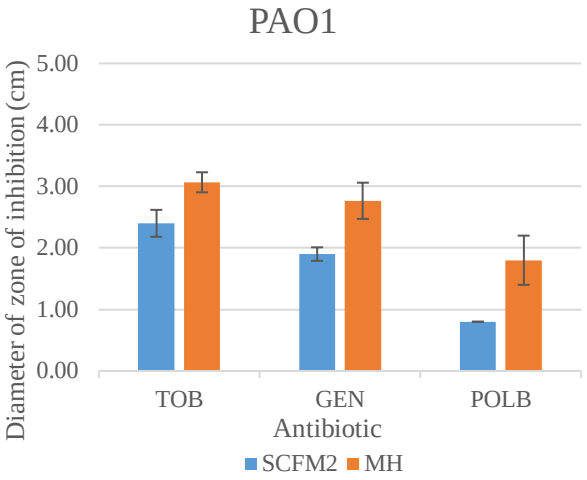


Figure 4 | PAO1 shows heightened resistance to all tested antibiotics in SCFM2 agar compared to MHA. An unpaired t test demonstrated differences in killing for all three antibiotics were significant (p = 0.0001). Zone of inhibition on MHA represented by orange bars and SCFM2 are represented by the blue bars. Error bars represent standard error of the mean. (n=3)

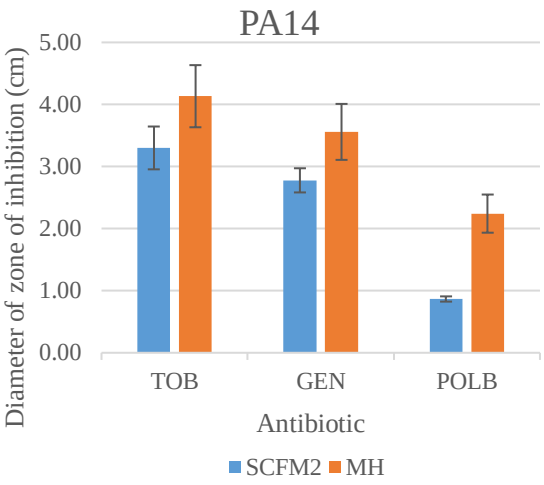


Figure 5 | PA14 shows heightened resistance to all tested antibiotics in SCFM2 agar compared to MHA. Results of an unpaired t test showed significance in the differences in killing of tobramycin (p = 0.0001), gentamicin (p = 0.0066), and polymyxin B (p =0.0038). Zone of inhibition on MHA represented by orange bars and SCFM2 are represented by the blue bars. Error bars represent standard error of the mean. (n=3)

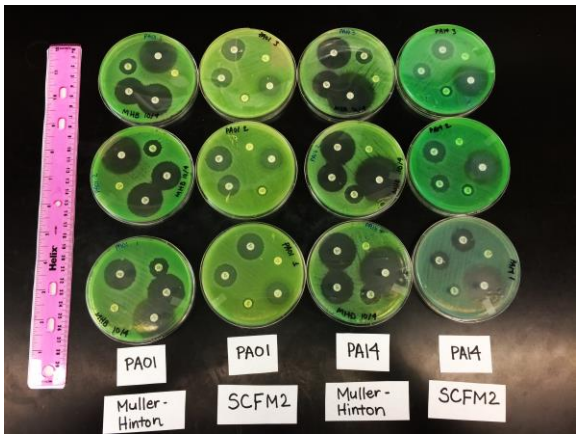


Figure 6 | Examples of disk diffusion assays performed on PA14 and PAO1 on SCFM2 and MHA. Measurements of the diameters of the kill zones were taken 24 hours after antibiotic disks were added. Experiments were replicated 3 times.

The clinical strains SED4, SED6, SED9, SED20, SED21—which were all isolated from a single patient—showed very different antimicrobial susceptibility profiles than the lab strains PA14 and PAO1. Although PA14 and PAO1 were more susceptible to all of the three antibiotics in the clinical diagnostic media, these strains did not mirror this pattern. All five strains were more susceptible to tobramycin in the SCFM2 agar than they were in the MHA. Additionally, these strains were unaffected by gentamicin in the MHA. However, when gentamicin disks were applied to these strains grown on SCFM2 agar, they were all susceptible to a similar degree. Most of the strains were more susceptible to polymyxin B in the MHA compared to the SCFM2 agar. The exception to this was strain SED21, which showed relatively equal levels of susceptibility in both types of media.

The British Society for Antimicrobial Chemotherapy (BSAC), sets the standards by which bacteria are classified as either resistant or susceptible to antimicrobials in the United Kingdom. As clinical isolates originated from and were initially characterized in the UK (Darch et al. 2015), we decided to continue to use these susceptibility 'breakpoints'. According to the BSAC standards for susceptibility to tobramycin, all five of these strains would be classified as susceptible to tobramycin when tested on MH, but resistant when tested on SCFM2.

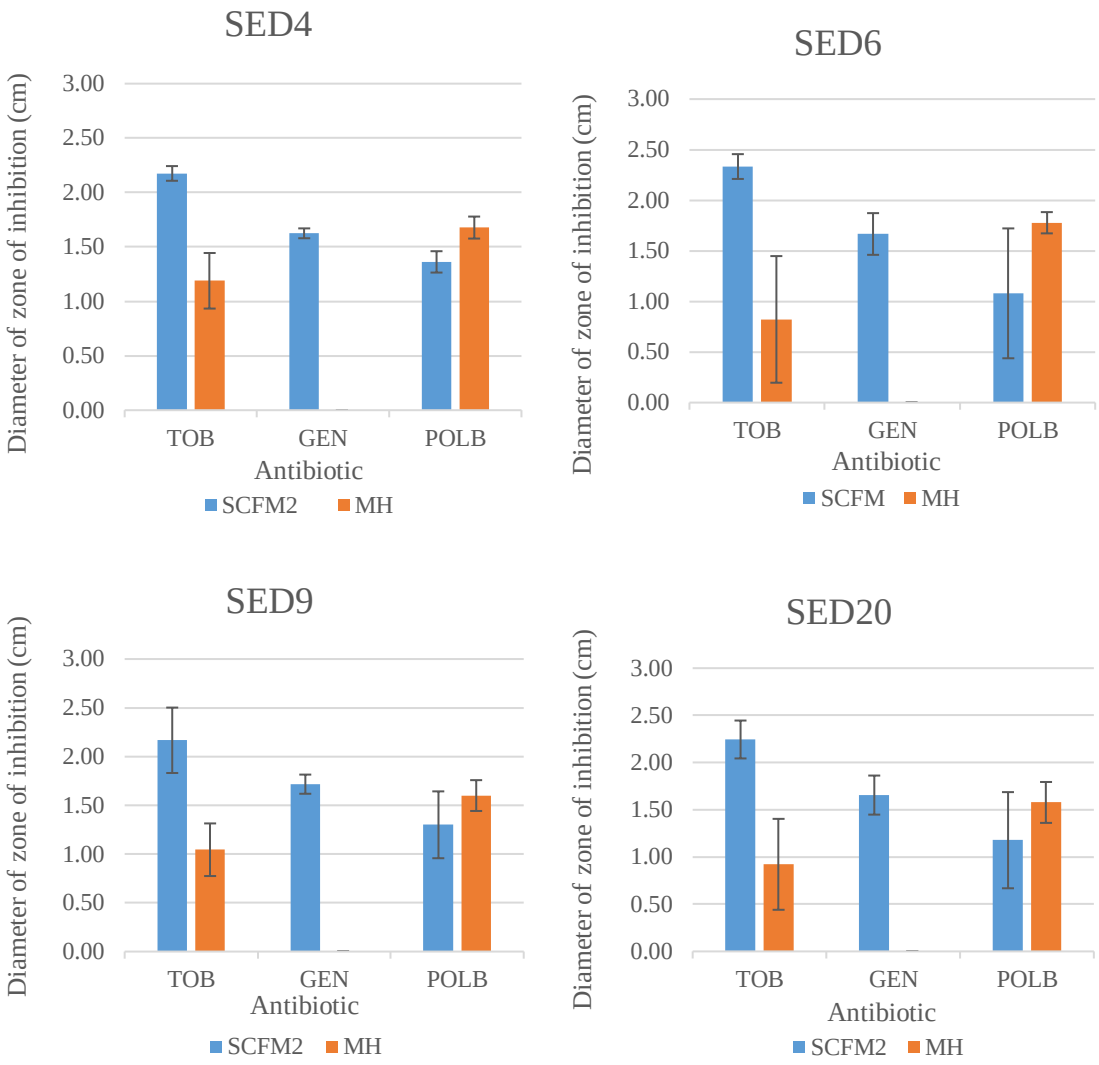


Figure 7 | Clinical strains SED4, SED6, SED9, SED 20, SED21 showed statistically significant different responses to tobramycin and polymyxin B. In SCFM2, SED4 was significantly more resistant to tobramycin ($p = 0.0030$) and less resistant to polymyxin B ($p = 0.0418$) as demonstrated by an unpaired t test. In the same medium, SED6 was significantly more resistant to tobramycin ($p = 0.0001$) and less resistant to polymyxin B ($p = 0.0015$) as demonstrated by an unpaired t test. Similarly, SED9 was significantly more resistant to tobramycin ($p = 0.0001$) and less resistant to polymyxin B ($p = 0.0298$) as demonstrated by an unpaired t test. Additionally, SED20 was significantly more resistant to tobramycin ($p = 0.0001$) and less resistant to polymyxin B ($p = 0.0456$) as demonstrated by an unpaired t test. Lastly, an unpaired t test showed that SED21 is significantly more resistant to tobramycin in SCFM2 ($p = 0.0001$). Zone of inhibition on MHA represented by orange bars and SCFM2 are represented by the blue bars. Error bars represent standard error of the mean. (n=3)

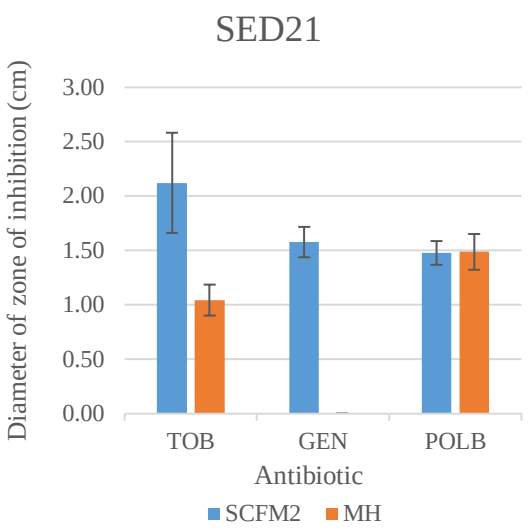
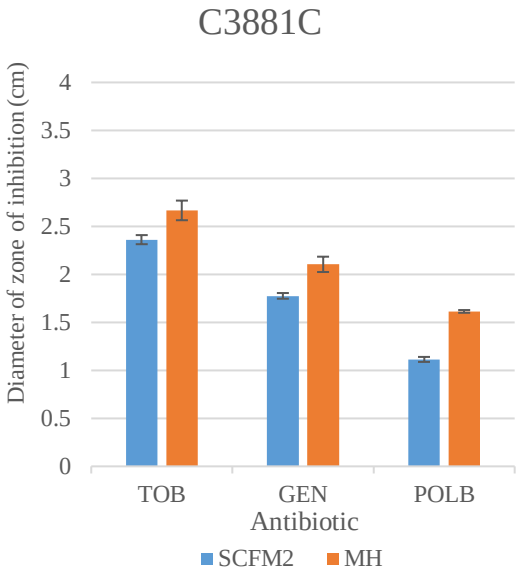
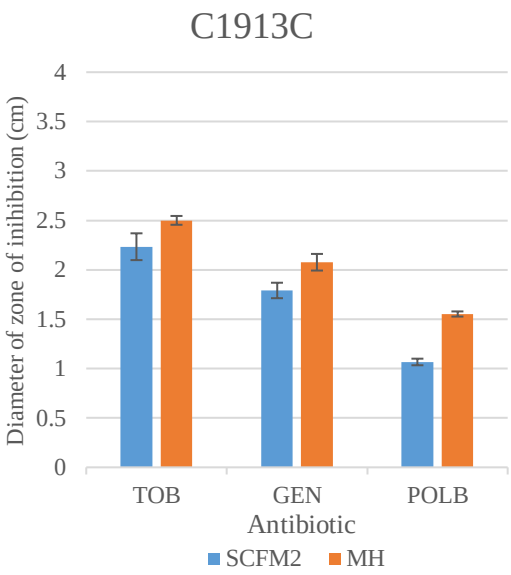


Figure 7 | Clinical strains SED4, SED6, SED9, SED20, and SED21 showed varying levels of resistance to the three antibiotics. Standard error of the mean was calculated and is denoted by the error bars. All strains were resistant to gentamicin in MHA (SEM ± 0). SED21 showed relatively equal levels of susceptibility to polymyxin B in SCFM2 (SEM ± 0.1093) and in MHA (SEM ± 0.1600). Zone of inhibition on MHA represented by orange bars and SCFM2 are represented by the blue bars. (n=3)

Three additional clinical strains—C2773C, C1913C, C3881C—were analyzed via disk diffusion assays. These clinical strains were isolated from three different patients and exhibit a mucoid phenotype, unlike the other seven strains that were tested. Like PAO1 and PA14, strains C1913C and C3881C were more resistant to most of the antibiotics when grown on SCFM2 agar than on MHA. According to an unpaired t test, strain C1913C did not show significantly higher levels of resistance to tobramycin on either growth medium ($p = 0.0562$). However, unlike the five clinical strains previously described, this strain was susceptible to gentamicin. When disks treated with this antibiotic were placed on C1913C grown on MHA, the zone of inhibition was 0.29cm wider and 15.96% greater than cells grown on SCFM2 agar. As expected, this strain was also susceptible to polymyxin B. When subjected to polymyxin B, C1913C grown on MHA had zones of inhibition that were 0.49cm wider and 45.54% greater than those produced around cells grown on SCFM2.

While strain C3881C showed fairly similar levels of susceptibility to these antibiotics on both media, strain C2773C had a completely different susceptibility profile. This strain was more susceptible to polymyxin B when the cells were grown on MHA than on SCFM2 agar. Cells that were tested on MHA had a zone of inhibition that was 0.67cm wider and 67.60% greater than those grown on SCFM2 agar. An unpaired t test demonstrated that this difference was significant (p=0.0001). Strain C2773C did not show significantly different responses to tobramycin or gentamicin depending on the growth medium in which it was cultured.





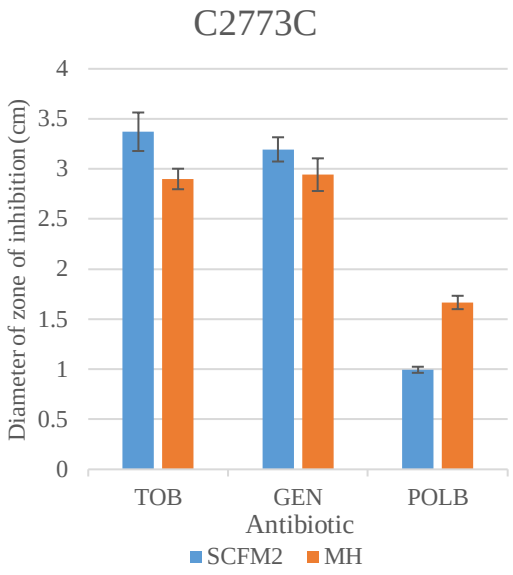
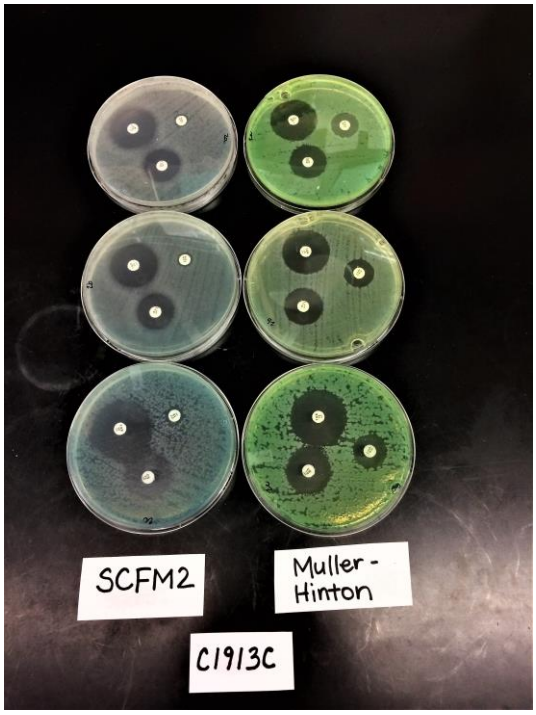


Figure 8 | Clinical strains C3881C, C1913C, and C2773C showed different susceptibility to some of the antibiotics depending on the medium the cells were tested in. As indicated by an unpaired t test, strain C3881C was significantly more resistant to tobramycin (p= 0.0192), gentamicin (p=0.0023), and polymyxin B (p=0.0001) in SCFM2 compared to MHA. In SCFM2, strain C1913C was only significantly more resistant to gentamicin (p= 0.0286) and polymyxin B (p= 0.0001). Strain C2773C was only significantly more resistant to polymyxin B (p= 0.0001). Zone of inhibition on MHA represented by orange bars and SCFM2 are represented by the blue bars. Error bars represent standard error of the mean. (n=3)



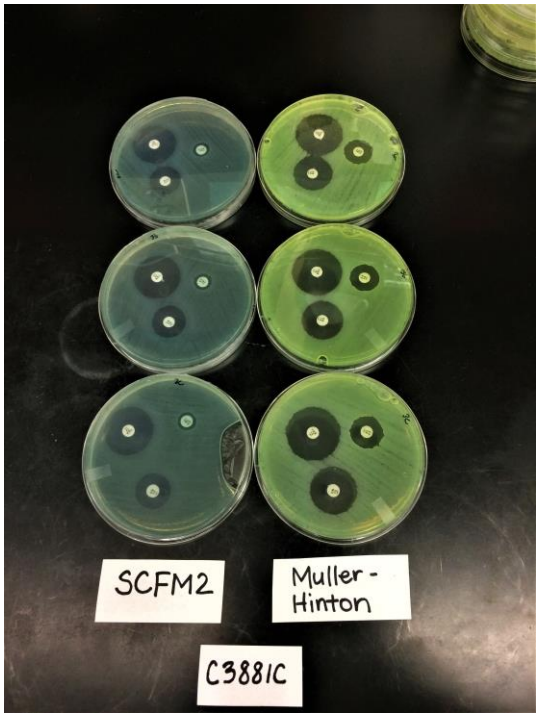


Figure 9 | Examples of disk diffusion assays performed on strains C1913C, C2773C, and C3881C. Measurements of the diameters of the kill zones were taken 24 hours after antibiotic disks were added. Experiments were replicated 3 times.

PA14 shows different levels of resistance in modified synthetic sputum media, standard synthetic sputum media, and clinical diagnostic media

SCFM2 is a chemically defined medium, meaning that components are known and can be readily added and subtracted during media preparation. In order to determine which components in SCFM2 may play a role in the resistance of PA14 to antibiotics compared to MH media, PA14 was tested in SCFM2 without sputum and SCFM2 without DNA. The results indicated that mucin was important for resistance to all three antibiotics, because when mucin was removed PA14 was more susceptible. In SCFM2 without DNA, PA14 was more susceptible to tobramycin and gentamicin, but not polymyxin B.

When mucin is removed, PA14 are significantly less resistant to tobramycin (**Figure 10a**) at concentrations 1.95e-3 μg/mL ($p<0.0001$), 3.91e-3μg/mL ($p<0.0001$), and 7.81e-3μg/mL ($p<0.0001$). The removal of mucin also impacted PA14 survival, as

measured by luminescence, when treated with gentamicin (**Figure 10b)**. PA14 was significantly less resistant to gentamicin at concentrations 1.95e-3 μg/mL ($p<0.0001$), 3.91e-3μg/mL ($p<0.0001$), and 7.81e-3μg/mL ($p<0.0001$). PA14 was also more susceptible to polymyxin B in SCFM2 without mucin (**Figure 10c**). This was observed at concentrations of 1.95e-3 μg/mL ($p<0.0001$), 3.91e-3μg/mL ($p=0.0005$), and 7.81e-3μg/mL ($p=0.0054$).

The removal of DNA also had an impact on PA14 survival when treated with tobramycin (**Figure 10a**) and polymyxin B (**Figure 10c**). When DNA was removed, PA14 was significantly less resistant to tobramycin at concentrations 3.91e-3μg/mL ($p=0.0287$) and 7.81e-3μg/mL ($p=0.0217$) and 1.56e-2μg/mL ($p= 0.0056$). Removal of DNA did not have a significant impact on the survival of PA14 treated with gentamicin (**Figure 10b**). However, removing DNA did have a significant on the survival of PA14 treated with polymyxin B. Without DNA, PA14 was significantly more susceptible to polymyxin B at concentrations 1.95e-3μg/mL ($p=0.0028$) and 3.91e-3μg/mL ($p=0.0110$).

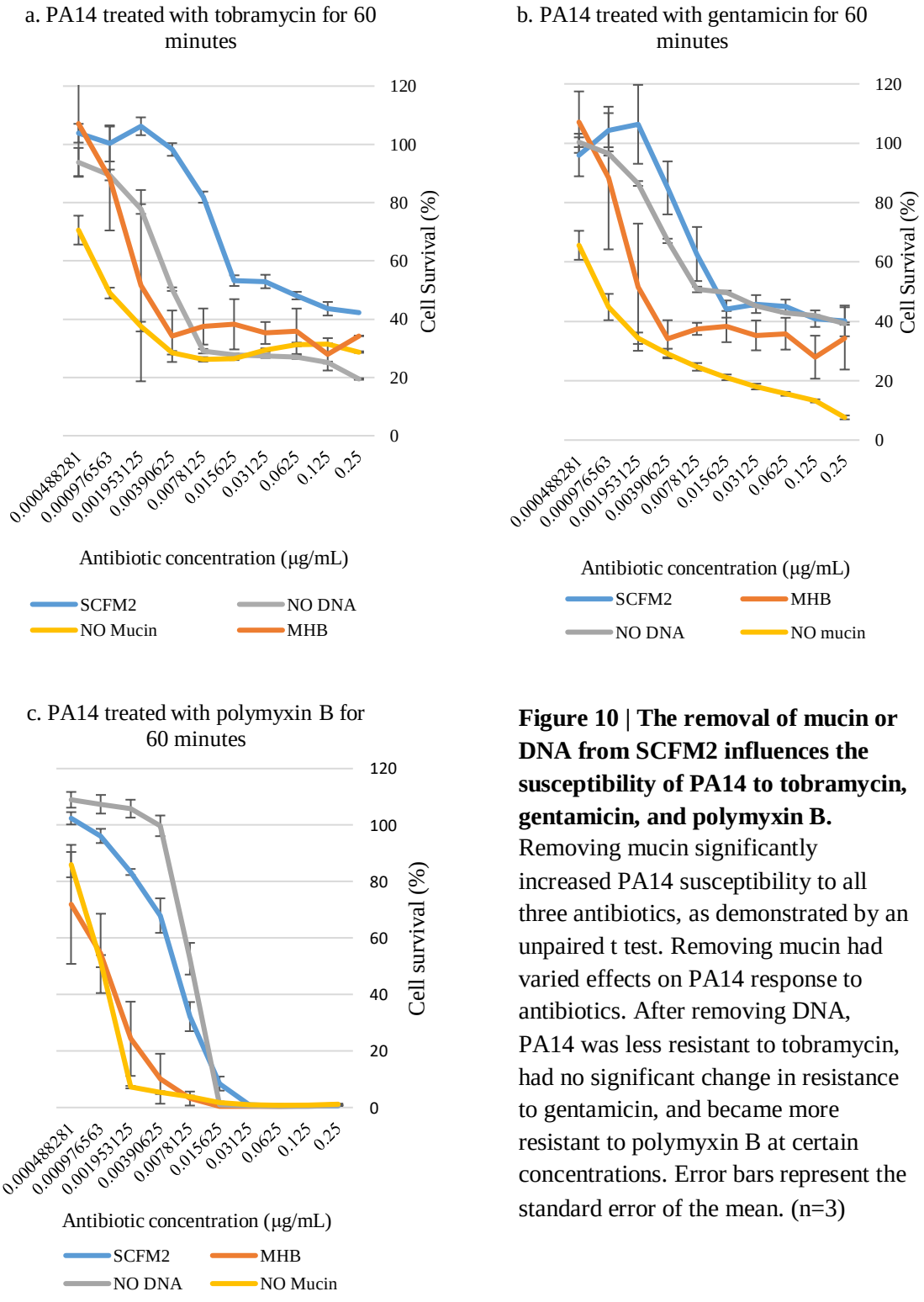


Figure 10 | The removal of mucin or DNA from SCFM2 influences the susceptibility of PA14 to tobramycin, gentamicin, and polymyxin B. Removing mucin significantly increased PA14 susceptibility to all three antibiotics, as demonstrated by an unpaired t test. Removing mucin had varied effects on PA14 response to antibiotics. After removing DNA, PA14 was less resistant to tobramycin, had no significant change in resistance to gentamicin, and became more resistant to polymyxin B at certain concentrations. Error bars represent the standard error of the mean. (n=3)

Section Four: Discussion

The goal of the study was to determine if testing the susceptibility of *P. aeruginosa* to different antibiotics in an environment that more closely recapitulates the CF lung would provide different results than tests conducted in nutritionally rich media used by diagnostic labs. The pathogen was evaluated using synthetic sputum media (SCFM2), which is nutritionally and physically similar to real CF sputum (Palmer et al., 2007). As such, it is known that the fitness requirements for growth of P. aeruginosa in this medium are nearly identical to real CF sputum (Turner et al., 2015). Ten different strains of P. aeruginosa were evaluated in SCFM2 and MH media, and the results showed that the pathogen demonstrated different levels of resistance to antibiotics depending on the media in which it was evaluated.

The use of SCFM2 in this manner is a novel, *in vitro* approach to evaluating the susceptibility of *P. aeruginosa* to antibiotics. Ideally, the utilization of SCFM2 in a diagnostic, clinical setting could help physicians to select more precise, individualized treatments for specific strains isolated from patients. In this study, it has demonstrated that different strains of *P. aeruginosa* respond differently to antibiotics depending on the growth medium in which the cells are cultured.

Previous studies on *P. aeruginosa* susceptibility to antibiotics have shown that there are a number of ways in which this bacterium is able to thwart antibiotic therapies. One key attribute is the ability of *P. aeruginosa* to form aggregates, and this has been demonstrated specifically in the CF lung (Kragh et al., 2016). Aggregation has previously been correlated with increased levels of antibiotic resistance (Connell, Wessel, 2012), although the underlying mechanisms behind this are not well understood. One theory lies in the fact that wildtype and some clinical strains of *P. aeruginosa* are known to produce

exopolysaccharides when aggregated, which are known to play a key role in protection from environmental disturbances, such as phage which target *P. aeruginosa* (Darch et al., 2017). It is possible that these exopolysaccharides could also play a key role in antibiotic resistance.

The minimum inhibitory concentration (MIC) assays revealed that strains PAO1 and PA14 were more resistant to three clinically relevant antibiotics in the synthetic sputum medium than in rich, MH medium. Since studies have shown that *P. aeruginosa* behaves similarly in SCFM2 to authentic CF lung sputum (Palmer et al., 2007; Turner et al., 2015), these results indicate that this pathogen could exhibit higher levels of resistance to antibiotics than current diagnostic testing techniques reveal. Therefore, in order to more accurately evaluate the antimicrobial susceptibility profile of *P. aeruginosa,* SCFM2 presents an alternative culture medium for the testing of isolates, rather than in rich media, such as MHB.

This study also highlights a role for mucin in the ability of *P. aeruginosa* to form aggregates, and/or protect aggregates from antibiotic treatment. On the MIC conducted in liquid SCFM2 which lacked mucin, *P. aeruginosa* was significantly more susceptible to all antibiotics. This indicates that mucin could function as a scaffold, facilitating aggregate and biofilm formation, or alternatively may act as a 'sink' for antibiotics, binding those that are freely available in the extracellular environment. In turn this would reduce their ability to enter a bacterial cell..

Disk diffusion assays were performed on ten different strains of *P. aeruginosa*. Like the MIC assays, PAO1 and PA14 showed higher levels of resistance to all three antibiotics on SCFM2 agar than on MHA. However, when this test was conducted on

eight clinical strains of *P. aeruginosa*, this was not always the case. The results from the disk diffusion assay showed that strains C2773C, SED4, SED6, SED9, SED20 and SED21 were more less susceptible to tobramycin and gentamicin on the MHA than on the SCFM2 agar. This indicates that assays conducted on rich media would produce results indicating that the bacteria are far more resistant to antibiotics than they actually would be in the patient's lungs. For instance, for strains SED4, SED6, SED9, SED20, and SED21, tests ran on rich media would show that a patient colonized with any of these strains could not be treated using gentamicin. However, when tested on SCFM2 agar, the tests reveal that this might not be the case.

In future experiments, MIC assays will be conducted on the eight clinical strains to ensure that the disk diffusion assays yield similar results to tests conducted in liquid media. These strains will be transformed with a plasmid carrying a light reporter, so that cell survival can be measured by the levels of light output. The strains can then be evaluated using the exact same protocol by which PAO1 and PA14 were tested.

The results of this study indicate that *P. aeruginosa* can have different levels of antimicrobial susceptibility in patients' lungs than in rich media used by diagnostic labs. Due to the level of severity that infections from *P. aeruginosa* can cause in the lungs of patients with Cystic Fibrosis, it is essential that improvements be made to the current methods used to evaluate the antimicrobial susceptibility of this pathogen. Prescribing ineffective antibiotics can lead to increased levels of resistance, making these infections almost impossible to remediate. However, it is known that with prompt, aggressive treatment, it is not impossible to eradicate the pathogen before it can cause serious harm to the patient (Frederiksen et al., 1997). This study shows that a small change to current

diagnostic methodology—changing the medium in which *P. aeruginosa* is evaluated—has the potential to provide CF patients with effective, personalized antibiotic therapies.

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Biography

Evelyn Abbott began her time at the University of Texas in the fall of 2012 majoring in Plan II Honors and History. After two years of study, she took a year off of college to teach English in Baku, Azerbaijan. After one year of working abroad, she returned to the University of Texas and decided to pursue a career in science. She decided to focus on microbiology, a began working at the Whiteley lab in the fall of 2015. Her research is centered around *P. aeruginosa* infections in the Cystic Fibrosis lung. She is third author on one scientific publication so far, which involved antibiotic and phage resistance of *P. aeruginosa.*

Evelyn Abbott was accepted into the University of Texas microbiology graduate program beginning in the fall 2017. Although there are a variety of research projects that she would like to pursue, she aims to focus on the microbial ecology of infections or environmental systems. She will finish her PhD sometime between 2023 and 2025. Afterwards, she hopes to secure a faculty position at a university and continue her research.

In her spare time, Evelyn Abbott enjoys traveling, camping, reading, and creative writing. She enjoys learning foreign language, and has studied Spanish, Russian, Turkish, and Azerbaijani. Her research paper entitled “Underrepresentation of African Americans in STEM” was accepted for publication in the Undergraduate Journal of Black Business History in the spring of 2017.